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Expression and DNA methylation of *TNF*, *IFNG* and *FOXP3* in colorectal cancer and their prognostic significance

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Background: Colorectal cancer (CRC) progression is associated with suppression of host cell-mediated immunity and local immune escape mechanisms. Our aim was to assess the immune function in terms of expression of *TNF*, *IFNG* and *FOXP3* in CRC.

Methods: Sixty patients with CRC and 15 matched controls were recruited. TaqMan quantitative PCR and methylation-specific PCR was performed for expression and DNA methylation analysis of *TNF*, *IFNG* and *FOXP3*. Survival analysis was performed over a median follow-up of 48 months.

Results: *TNF* was suppressed in tumour and *IFNG* was suppressed in peripheral blood mononuclear cells (PBMCs) of patients with CRC. Tumours showed enhanced expression of *FOXP3* and was significantly higher when tumour size was >38 mm (median tumour size; $P=0.006$, Mann–Whitney *U*-test). Peripheral blood mononuclear cell *IFNG* was suppressed in recurrent CRC ($P=0.01$). Methylated *TNFpromoter* ($P=0.003$) and *TNFexon1* ($P=0.001$) were associated with significant suppression of *TNF* in tumours. Methylated *FOXP3cpg* was associated with significant suppression of *FOXP3* in both PBMC ($P=0.018$) and tumours ($P=0.010$). Reduced PBMC *FOXP3* expression was associated with significantly worse overall survival (HR = 8.319, $P=0.019$).

Conclusions: We have detected changes in the expression of immunomodulatory genes that could act as biomarkers for prognosis and future immunotherapeutic strategies.

Patients with colorectal cancer (CRC) are likely to be immunosuppressed due to a number of factors including older age (Pawelec *et al*, 2002) and poor nutritional status (Grunfeld, 2002). However, CRC itself has a direct suppressive effect on the cell-mediated immunity (CMI) as demonstrated by studies showing resolution of normal immunological function after successful tumour resection (Heriot *et al*, 2000; Galizia *et al*, 2002). A shift in immune function has been demonstrated in patients with CRC. Decreased total numbers of TH1 CD4+ cells have been found in patients with CRCs (Nakayama *et al*, 2000). There is also reduced production of cytokines from TH1 lymphocytes(TNF-α and IFN-γ) while those produced by TH2 lymphocytes appear to remain at normal or even elevated levels (O'Hara *et al*, 1998; Heriot *et al*, 2000; Shibata *et al*, 2002; Kanazawa *et al*, 2005). TNF-α and IFN-γ levels were shown to be particularly low in CRC with vascular invasion (Evans *et al*, 2010).

Tumour necrosis factor-α (TNF-α) is a TH1 cytokine with both pro- and anti-cancer properties. Multiple experiments have shown that TNF-α produced chronically at low picogram levels in the tumour microenvironment, whether by tumour or stromal cells (or most likely both) may cause direct DNA damage, may have anti-apoptotic or mitogenic activity, may mediate tumour/stromal cell interactions and induce a range of matrix metalloproteinases, cytokines and chemokines that promote tumour development (Balkwill, 2006). Activation of regulatory T cells (Tregs) can cause immunosuppression and has resulted from prolonged exposure to

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TNF-α (Chen *et al*, 2007), which could have a cancer promoting effect. On the contrary, *in vivo* recombinant TNF-α directly injected into tumours destroys the tumour vasculature (Watanabe *et al*, 1988). Genetically engineered tumour cells producing high levels of TNF-α have been implanted into tumours and, although they do not kill the tumours, they inhibit the growth through the activation of macrophages and natural killer cells (Blankenstein *et al*, 1991). Heriot *et al* (2000) have shown a highly significant reduction in production of TNF-α and IFN-γ (interferon-γ) by lipopolysachharide (LPS) stimulated whole-blood cultures derived from patients with CRC as compared to similarly aged controls. They had also shown that suppression of TNF-α and IFN-γ production was higher in late-stage tumours compared to early-stage tumours and this disappears following the resection of tumour. A subsequent follow-up study confirmed that patients who had lower levels of TNF-α and IFN-γ had significantly worse survival (Evans *et al*, 2006).

T-regulatory cells are implicated in the development of autoimmunity, allergy and rejection of organ transplants, as well as the suppression of immune responses to cancer. There is an increased presence of CD4 + CD25 + T cells in a wide spectrum of human malignancies, such as lung, head and neck, ovarian, gastrointestinal and skin. These cells are found in relatively high concentrations in blood, ascites, tumour draining lymph nodes and within the tumour milieu of cancer patients. Regulatory T cells were initially characterised by the CD4 + CD25 + phenotype and are thought to modulate the antitumour immune response (Zou, 2006; Curiel, 2007). Depletion of intratumoural Tregs was found to enhance antitumour immunity and tumour rejection in mouse models (Needham *et al*, 2006). Loddenkemper *et al* (2006) reported that Treg density in human CRC was lower in node-positive disease but was not associated with survival. However, Ling *et al* (2007) found no significant difference in Treg density between advanced- and early-stage disease.

The most specific Treg-cell marker identified is the nuclear transcription factor known as *FOXP3* (Fontenot *et al*, 2003; Hori *et al*, 2003). A high density of tumour-infiltrating FOXP3 + Tregs has been associated with poor outcome in various solid tumours, including ovarian (Curiel *et al*, 2004; Sato *et al*, 2005), pancreatic (Hiraoka *et al*, 2006) and hepatocellular carcinoma (Kobayashi *et al*, 2007). The expression of *FOXP3* in naive CD4 + T cells has been shown to decrease IFN-γ and IL-2 secretion and leads to increased expression of other receptors, which are characteristic of Tregs, such as CD25, glucocorticoid-induced TNF receptor and cytotoxic T Lymphocytic Antigen 4 (Hori *et al*, 2003). The importance of *FOXP3* in functioning Tregs is now well established and is thought to be induced by TCR activation in conjunction with TGF-β stimulation (Coffer and Burgering, 2004). Moreover, *FOXP3* expression has also been seen in tumour cells, which may also provide tumours with direct immunosuppressive powers (Hinz *et al*, 2007; Ebert *et al*, 2008). The Tregs downregulating tumour-specific immunity is particularly prominent in solid tumours such as colorectal carcinoma (Curiel *et al*, 2004). *FOXP3* expression mediated by cancer cells have been shown to contribute towards disease progression in CRC (Kim *et al*, 2013). As FOXP3 is a crucial regulator of T regs, compounds that inhibit the expression, function and signalling of FOXP3 might have therapeutic potential.

Published reports from our department have demonstrated the significance of TNF-α and IFN-γ levels in CRC (Heriot *et al*, 2000; Evans *et al*, 2006; Evans *et al*, 2010). In line with the literature (Somasundaram *et al*, 2002; Wolf *et al*, 2003) unpublished research in our department has shown increased numbers of Treg cells in CRC. With a view to take these findings forward, our aim was to assess the immune function in terms of the expression of *TNF*, *IFNG* and *FOXP3* in peripheral blood and tumour of patients with CRC. We also aimed at assessing the influence of DNA methylation on the expression of the study genes, which would be helpful in identifying a potential role for epigenetic regulation in CRC.

MATERIALS AND METHODS

A total of 60 patients with a diagnosis of colorectal adenocarcinoma were recruited into the study following approval by the local research ethics committee (South West London REC 3, UK). Peripheral blood mononuclear cells (PBMCs) were separated from venous blood using Ficoll–Hypaque density gradient method. Total RNA and DNA were isolated from the PBMCs, fresh frozen tumour tissue and normal mucosa (Qiagen AllPrep DNA/RNA Mini kit and QIAshredder), and simultaneous purification of genomic DNA and total RNA was performed from a single biological sample. The total RNA and DNA extracted from all the samples were analysed for quantity and quality using the NanoDrop spectrophotometer. RNA and DNA extraction were repeated for samples that did not meet the manufacturer's recommendations. The samples that were not satisfactory for either quantity or quality during repeat extraction were excluded from the analysis.

TaqMan quantitative PCR (qRT-PCR, two step) was used to determine the relative fold change in the expression of *TNF*, *IFNG* and *FOXP3* in the PBMC (compared to controls) and tumour (compared to normal mucosa), with *GAPDH* as internal control. The total RNA isolated was reverse transcribed into cDNA using the High Capacity cDNA Reverse Transcription kit (Applied Biosystems, Foster City, CA, USA). Predesigned TaqMan Gene Expression Assays (Applied Biosystems) for the test (assay ID: Hs00174128_m1 – *TNF*, Hs00989291_m1 – *IFNG*, Hs01085834_m1 – *FOXP3*) and reference gene (*GAPDH*) were obtained from Applied Biosystems. Quantitative analysis of the PCR products were performed using the ABI 7500 Fast Real-time PCR system (Applied Biosystems). Relative quantification of gene expression was performed using the $2^{-\Delta\Delta C_t}$ method (Livak and Schmittgen, 2001).

Methylation-specific PCR (MSPCR) was performed to identify the methylation status of the study genes. EZ-96 DNA Methylation-Gold kit (Zymo Research, Irvine, CA, USA) was used for the bisulfite conversion of DNA. Subsequently a multiplex PCR was performed. Each 15 μl MSPCR included 7.5 μl Qiagen Multiplex Master Mix (Qiagen, Hilden, Germany) consisting of HotStarTaq DNA polymerase (Qiagen), multiplex PCR buffer, Mg^{2+} and dNTPs at optimum concentration; 3 μl Q solution (Qiagen), 0.3 μl each of forward and reverse primers, 2.9 μl of RNAse-free water and 1 μl of bisulfite-converted template DNA. All PCRs were undertaken in 96-well PCR plates (ABgene, Thermo Scientific, Waltham, MA, USA). The PCR conditions were hot-start step of 95°C for 15 min, followed by a 35-cycle programme consisting of 94°C for 60 s, 60°C for 90 s, 72°C for 1 min followed by final 72°C for 10-min extension step. The primer sequences used in the MSPCR are enumerated in Table 1. MSPCR products were analysed on agarose gel electrophoresis. Methyltransferase-treated and -untreated DNA was used as controls for methylated and unmethylated DNA. Samples were defined as methylated or unmethylated depending upon the visual band amplified with methylated or unmethylated primers. Microsatellite instability (MSI) status of the tumour samples were analysed by PCR using microsatellite primers BAT25 and BAT26.

The control group included 15 matched patients who attended colorectal clinic with benign colorectal disease and underwent investigations, which ruled out CRC. The control group was used only for the comparison of expression of study genes in PBMC samples. All data analyses were performed using the SPSS 19.0 software (SPSS, Chicago, IL, USA). Pearson's chi-square analysis and Fisher's exact test were employed to compare the difference of

Table 1. The primer sequences used for MSPCR, obtained from Sigma-Aldrich

Name	Sequence
TNF-pr-Meth-F	TAGAAGGTGTAGGGTTTATTATCGT
TNF-pr-Meth-R	TACCTTTATATATCCCTAAAACGAA
TNF-pr-Unmeth-F	TAGAAGGTGTAGGGTTTATTATTGT
TNF-pr-Unmeth-R	TACCTTTATATATCCCTAAAACAAA
TNF-ex-Meth-F	GAGTATTGAAAGTATGATTCGGGAC
TNF-ex-Meth-R	CAACAAACAAAAAAACGTAATAACG
TNF-ex-Unmeth-F	GTATTGAAAGTATGATTTGGGATGT
TNF-ex-Unmeth-R	ACAAACAAAAAAACATAATAACACC
IFNg-Meth-F	GTGGGTATAATGGGTTTGTTTTATC
IFNg-Meth-R	AATTAAAATCTCCTAAAAATTACGTA
IFNg-Unmeth-F	GGTATAATGGGTTTGTTTTATTGT
IFNg-Unmeth-R	AATTAAAATCTCCTAAAAATTACATA
FOXP3-enc-Meth-F	GTAAAGGGTAGTTGGAAGGTAAAGC
FOXP3-enc-Meth-R	GTACGAACCTCACACGACGA
FOXP3-enc-Unmeth-F	TAAAGGGTAGTTGGAAGGTAAAGTG
FOXp3-enc-Unmeth-R	TACACATACAAACCTCACACAACAA
FOXP3-CpG-Meth-F	AGAGGTTTAAAAAGTGGGAGATTTC
FOXP3-CpG-Meth-R	ATTAACTCGCTACAACCATTATCGT
FOXP3-CpG-Unmeth-F	AGAGGTTTAAAAAGTGGGAGATTTT
FOXP3-CpG-Unmeth-R	TTAACTCACTACAACCATTATCATC

Abbreviations: enc = enhancer; ex = exon1; F = forward; Meth = methylated; pr = promoter; R = reverse; Unmeth = unmethylated.

Table 2. The gene expression levels relative to GAPDH in our study samples

	Median fold change	Range	*P* value (mann-whitney)
TNF PBMC	0.89	0.05–82.87	0.908
TNF tumour	**0.48**	0.01–14.48	**0.005**
IFNG PBMC	**0.34**	0.02–23.55	**<0.001**
IFNG tumour	1.21	0.04–57.63	0.613
FOXP3 PBMC	**0.24**	0.07–33.53	**<0.001**
FOXP3 tumour	**2.20**	0.05–79.73	**0.043**

Abbreviation: PBMC = peripheral blood mononuclear cell. Bold indicates statistically significant values.

categorical variables between patient groups. The relative gene expression levels were compared between various subgroups using Kruskal–Wallis test (>2 groups) and Mann–Whitney *U*-test (two groups). Survival was analysed according to Kaplan–Meier method. Significance was assumed when the statistical tests returned *P*-values <0.05.

RESULTS

The study group included 60 patients (32 males, 28 females); 68% ($n=41$) of patients had left-sided tumours (at or distal to splenic flexure), which were predominantly rectal ($n=27$). Two patients had synchronous tumours in the rectum and sigmoid colon. The study group included seven patients with recurrent cancers, the sites of which included rectum ($n=5$), caecum ($n=1$) and left paracolic space ($n=1$). Tumour size varied from 5 to 140 mm (mean 44.8, median 38 and s.d. 23.1). Tumour VI was present in 32% ($n=18$) of our patients and 18% ($n=11$) had poor differentiation. The TNM staging distribution of patients with primary CRC was – stage 1 ($n=9$), stage 2 ($n=22$), stage 3 ($n=18$) and stage 4 ($n=4$). Among the patients with recurrent CRC ($n=7$) the primary staging distribution was – stage 2 ($n=3$), stage 3 ($n=1$) and stage 4 ($n=3$). Supplementary Table 1 enumerates the patient and tumour characteristics.

Gene expression – qRT-PCR. The gene expression levels as measured by the relative quantification method for our study genes are enumerated in Table 2. Gene expression levels are relative to *GAPDH* expression where *GAPDH* expression level is equal to 1. Although the median expression levels of the *TNF* in the PBMC samples were close to reference level (median 0.89), the *TNF* was found to be suppressed in the tumour samples (median 0.48). On the contrary *IFNG* was found to be suppressed in the PBMC samples (median 0.34), but the expression levels in the tumour samples were close to the reference level (median 1.21). Although the expression levels of *FOXP3* were reduced in the PBMC samples (median 0.24), increased expression was seen with tumour samples (median 2.20).

Gene expression and tumour characteristics. Although the expression of *TNF* in tumours were suppressed as mentioned above, the suppression was found to be maximum in stage 4 tumours and was statistically significant ($P=0.04$; Figure 1A). No significant difference was noticed with respect to *TNF* expression in the PBMC based on stage. However, significant suppression of the PBMC *IFNG* was noticed in stage 4 tumours ($P=0.01$; Figure 1B). *TNF* expression in the tumour samples was significantly high in patients with VI ($P=0.034$; Supplementary Figure 1). *IFNG* suppression was significant in the recurrent CRC group compared to the primary CRC group ($P=0.01$; Supplementary Figure 2). *FOXP3* expression in tumours was found to be high in stage 3 tumours, however, the enhanced expression approached but did not reach statistical significance ($P=0.06$). Although there was an enhanced expression of *FOXP3* in tumours, a significantly increased expression was particularly noticed in large-sized tumours (>38 mm, median tumour size = 38 mm, $P=0.006$; Figure 2). A significant positive correlation was noticed in the expression levels of *TNF* and *IFNG* in both PBMC ($P=0.001$) and tumours ($P=0.006$). The gene expression levels did not have any significant association with grade of CRC.

DNA methylation and gene expression. Samples were defined as methylated or unmethylated depending upon the visual band amplified with methylated or unmethylated primers (Figure 3). The methylation status and expression levels with respect to the study genes are enumerated in Table 3. *TNF* methylation was examined at the promoter region and the first exon. *TNF* expression levels were analysed in the two subgroups (methylated and unmethylated). A significant difference was observed in tumour samples with lower expression found in the methylated group compared to the unmethylated group at both *TNFpromoter* ($P=0.003$) and *TNFexon1* ($P=0.001$) sites. The *TNF* expression in the PBMC samples did not have a significant association with methylation status. The DNA methylation status of *TNFpromoter* and first exon in both tumours and PBMC did not have any significant association with other pathological prognostic factors.

IFNG promoter methylation was observed in 65% of PBMC samples and 55% of tumour samples in patients with CRC. There was no significant difference observed in the expression levels between the methylated and unmethylated groups. There was no

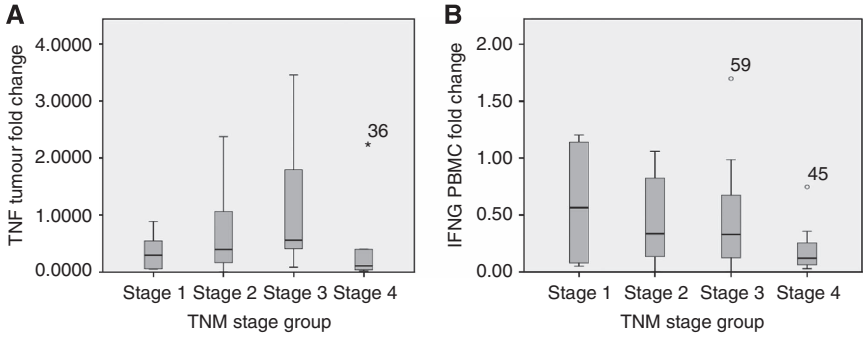

Figure 1. (**A** and **B**) Levels of TNF and IFNG expression significantly reduced in stage 4 disease in tumour and PBMC samples, respectively (36, 59 and 45 indicate outlier sample numbers).

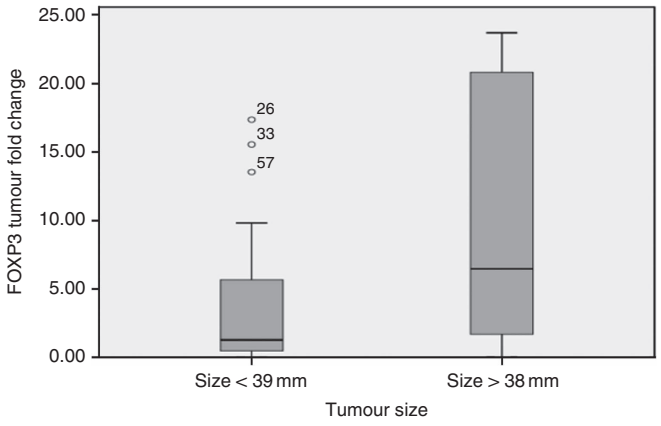

Figure 2. Levels of expression of FOXP3 in tumour tissue was significantly enhanced in large tumours (>38 mm, median tumour size) (26, 33 and 57 indicate outlier sample numbers).

significant association between the methylation status of the *IFNG* and the relative *IFNG* expression level. The methylation status of *IFNG* in both PBMC and tumour samples did not have any significant association with any of the pathological prognostic indicators.

FOXP3 methylation was analysed at enhancer and CpG island sites. *FOXP3cpg* methylation was observed in 70% of PBMC samples and 39% of tumour samples in patients with CRC. *FOXP3* relative expression levels were analysed in the two subgroups (methylated and unmethylated). There was a significant difference in the levels of FOXP3 expression with lower expression in the methylated group in both PBMC ($P=0.001$) and tumour samples ($P=0.010$). *FOXP3enc* methylation status was not associated with significant difference in the expression levels. There was no correlation noted with *FOXP3* methylation status and tumour size even though the tumour size correlated with relative *FOXP3* expression.

Microsatellite instability status, gene expression and DNA methylation. Microsatellite instability status was analysed in 47 patients in our study; 19% (9 of 47, 4 males) of patients in our study were found to have MSI. Although 14% of males with CRC were found to have tumours positive for MSI, 26% of females were found to be positive for MSI. However, the difference was not statistically significant; 9% (4 of 47) were found to have MSI at both BAT25 and BAT26 loci, while the other five patients were positive in either of the two loci. Significant *TNF* suppression ($P=0.002$) was noted in the PBMC samples of patients with MSI compared to patients who were microsatellite stable (MSS; Supplementary Figure 3). However, the TNF expression in the tumour samples did not correlate with MSI status. There was no significant difference noted in the expression of *IFNG* and *FOXP3* between MSI and MSS patients. There was also no correlation observed between the MSI status and DNA methylation status of our study genes.

Survival analysis. During a median follow-up period of 48 months a total of 23 patients died. Among the patients who underwent curative resection of CRC, 14 patients developed recurrent disease. In order to analyse the survival based on relative gene expression, patients were classified into two groups based on the median expression level. Patients who had higher *FOXP3* expression in the PBMC samples had a significantly better overall survival (OS) compared to patients with lower *FOXP3* expression ($P=0.022$, log-rank test; Figure 4A). Similarly, patients with higher PBMC *FOXP3* expression had a significantly better recurrence-free survival (RFS, $P=0.001$; Figure 4B). There was no significant difference in OS and RFS noted with respect to the expression of *TNF* and *IFNG* in both PBMC and tumour samples. On multivariate Cox regression analysis (Table 4) PBMC *FOXP3* expression was found to be a significant factor influencing the OS (HR = 8.319, $P=0.019$; Supplementary Figure 4) and to a lesser extent the RFS (HR = 5.106, $P=0.173$).

DISCUSSION

The median expression levels of the genes TNF (median 0.48) and IFNG (median 0.34) were found to be low in the tumour and PBMC samples, respectively. It has been demonstrated in the past that the production of TNF-α and IFN-γ by LPS stimulated whole-blood cultures derived from patients with CRC are reduced as compared to controls (Heriot *et al*, 2000). It was also shown that the suppression of TNF-α and IFN-γ cytokine production was higher in the late stages than the earlier stages of CRC. In this study, we have shown that the suppression of TNF-α cytokine levels is likely to be due to the suppression of *TNF* gene in the tumour microenvironment rather than systemic. However, low levels of *IFNG* expression was noted in the PBMC samples in our study, which indicate that the site of *IFNG* suppression is likely to be systemic rather than in the tumour microenvironment. Although no specific pattern with *TNF* and *IFNG* suppression was noted with respect to stages 1–3 CRC, suppression was found to be statistically significant in stage 4 disease. Although it is possible that immune suppression as represented by the reduced levels of expression of CMI-associated cytokine genes *TNF* and *IFNG* could be the reason behind progression of the disease to stage 4, this study is able to establish the correlation but unable to

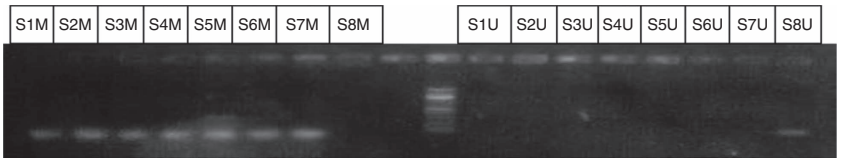

Figure 3. Agarose gel electrophoresis demonstrating the MSPCR products for a series of eight tumour samples with TNF methylated primers on the left and TNF unmethylated primers on the right of 100 bp ladder. Although the first seven samples showed methylated product, sample 8 showed unmethylated product. Abbreviations: S1M = sample 1 methylated; S1U = sample 1 unmethylated.

Table 3. The methylation status of samples illustrated at various sites

Site	Sample	Methylation status	Expression median(min-max)	*P* value
TNFpromoter	PBMC	M – 55%	0.69(0.06–82.87)	0.561
		U – 45%	1.23(0.32–14.89)	
TNFpromoter	Tumour	M – 67%	0.31(0.02–10.44)	0.003
		U – 33%	1.87(0.56–14.48)	
TNFexon1	PBMC	M – 59%	0.83(0.06–82.87)	0.894
		U – 41%	1.03(0.36–16.26)	
TNFexon1	Tumour	M – 67%	0.24(0.02–1.55)	0.001
		U – 33%	2.18(0.48–14.48)	
IFNGpromoter	PBMC	M – 65%	0.14(0.01–23.56)	0.533
		U – 35%	0.87(0.02–11.82)	
IFNGpromoter	Tumour	M – 55%	0.61(0.01–30.55)	0.176
		U – 45%	2.73(0.55–157.63)	
FOXP3cpg	PBMC	M – 70%	0.16(0.01–0.55)	0.001
		U – 30%	0.52(0.15–33.53)	
FOXP3cpg	Tumour	M – 39%	0.72(0.01–20.75)	0.010
		U – 61%	9.86(0.01–78.21)	
FOXP3enc	PBMC	M – 67%	0.25(0.01–0.55)	0.051
		U – 33%	0.36(0.01–33.53)	
FOXP3enc	Tumour	M – 37%	0.72(0.01–20.75)	0.054
		U – 63%	6.76(0.01–78.21)	

Abbreviation: PBMC = peripheral blood mononuclear cell. The expression levels of methylated and unmethylated samples are compared (Independent samples test; M-Methylated, U-Unmethylated).

confirm the cause and effect relationship. Similar to stage 4 disease, presentation of patients with recurrent disease following previous resection also represents disease progression. We also noticed that the expression levels of *IFNG* in the PBMC samples were significantly low in recurrent CRC. The early evidence for antitumour effect of IFNs came from carcinogenesis experiments employing mice that lack IFN-γ, where spontaneous tumour development was more frequent and rapid as compared to wild-type mice. Interferon-γ is an important antiproliferative cytokine that is involved in multiple pathways to check the tumour progression (Brandacher *et al*, 2006). It is possible that immuno-suppression in the form of low IFN-γ levels could have contributed

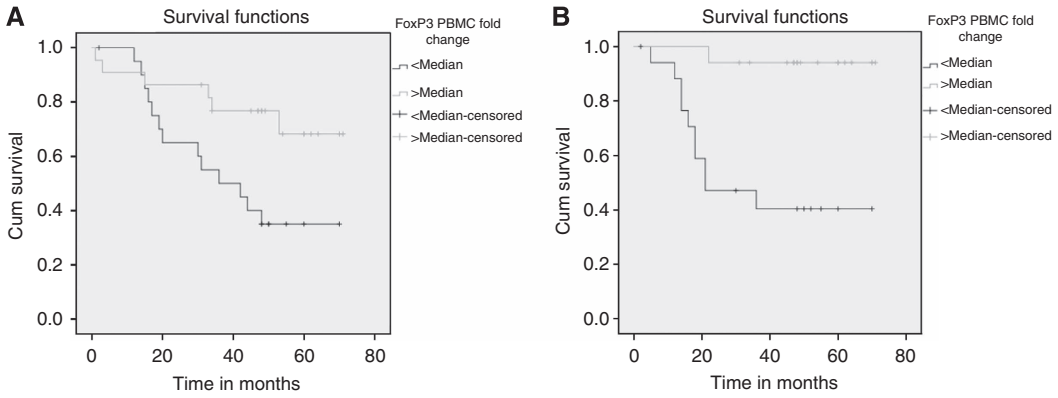

Figure 4. (**A** and **B**) Kaplan–Meier survival curves for OS ($P = 0.022$, **A**) and RFS ($P = 0.001$, **B**) in patients classified based on the median expression levels of PBMC FOXP3. Abbreviation: Cum = cumulative.

Table 4. Multivariate Cox regression analysis of various factors influencing the OS

	B	SE	Sig	HR	95% CI for exp(B)	
					Lower	Upper
Age	-0.864	0.651	0.184	0.421	0.118	1.508
Stage	1.473	1.012	0.145	4.363	0.030	3.275
T status	–1.154	1.194	0.334	0.316	0.577	21.120
N status	1.251	0.918	0.173	3.492	0.162	4.463
Grade	–0.162	0.846	0.848	0.850	0.080	1.704
Vascular invasion	–0.995	0.780	0.202	0.370	1.415	48.917
PBMC FOXP3 expression	2.119	0.904	0.019	8.319	0.601	31.684

Abbreviations: B = regression coefficient; CI = confidence interval; exp(B) = hazard ratio; HR = hazard ratio; PBMC = peripheral blood mononuclear cell; SE = standard error; Sig = significance.

to the recurrence of CRC. We feel that the disease progression in the form of recurrent disease in CRC is likely to be due to the systemic immune suppression as represented by low IFNG expression in PBMC. We have demonstrated a significant positive correlation between the expression of TNF and IFNG, reiterating the fact that both these cytokines act through TH1 pathways that are associated with immune deviation towards enhanced or suppressed tumour rejection based on their activity.

Vascular invasion is seen as one of the discrete steps in the metastatic cascade and results in tumours being capable of shedding millions of tumour cells into the vascular circulation (Liotta *et al*, 1974). Vascular invasion has been shown to be an independent predictor for haematogenous disease recurrence and decreased survival in CRC (Talbot *et al*, 1980; Harrison *et al*, 1994; Petersen *et al*, 2002; Morris *et al*, 2006; Quirke and Morris, 2007; Sato *et al*, 2010). We found that the expression of TNF in tumours was significantly high in the presence of VI. An earlier study found that serum levels of TNF-α was significantly low in the presence of VI (Evans *et al*, 2010). It is possible that the presence of VI leads to systemic tumour exposure and may explain the significant fall in systemic TH1 cytokine production. Increased expression of TNF in the tumours with VI in our study could be a manifestation of cancer promoting effect of TNF. It is well known that TNF-α is a TH1 cytokine with both pro- and anticancer properties. In prostate cancer, TNF has been shown to enhance the *in vitro* migration and invasion of tumour cells through increasing the expression of several glycosyl- and sulfo-transferase genes that are involved in the synthesis of mucin-type selectin ligands (Radhakrishnan *et al*, 2011). As a result, TNF enhanced the entry of tumour cells into the bloodstream.

DNA methylation is an important regulator of gene transcription, and its role in carcinogenesis has been a topic of considerable

interest in the last few years. It is currently well known that cancer is characterised by a tremendous amount of epigenetic aberrations, and hence it could be postulated that these aberrations could have an influence on the immune genes in cancer. Hence the DNA methylation status was analysed in our samples in order to correlate it with the expression levels. The significantly lower expression of *TNF* in methylated tumours illustrates a likely role of DNA methylation in silencing the *TNF* gene in CRC. Through CRC cell-line experiments Sullivan *et al* (2007) had shown the possibility of a direct role for DNA methylation in the regulation of TNF-α expression. The influence of DNA methylation on the expression of *TNF* in human CRC has not been studied in the past. However, we did not find a significant correlation between the methylation status of the *IFNG* and the relative *IFNG* expression level. A strong correlation was observed in the DNA methylation status and expression of FOXP3 in both PBMC and tumour samples. With the help of murine experiments Polansky *et al* (2008)had shown that DNA methylation in T-regulatory cell-specific demethylated region not only regulates *FOXP3* gene transcription, but also is critically involved in maintaining stable *FOXP3* expression. Hence our finding of a strong correlation illustrates the possible role for DNA methylation in the regulation of *FOXP3* in human CRC.

Over the past few decades it has become clear that CRC evolves through multiple pathways and that these pathways can be roughly defined on the basis of molecular patterns such as the integrity of the mismatch repair system or mutational and epigenetic patterns. Tumours with MSI have greater numbers of tumour-infiltrating lymphocytes that are activated and cytotoxic (Phillips *et al*, 2004) and the lymphocytic reaction is independently associated with longer survival (Ogino *et al*, 2009). Hence, we classified the patients based on their MSI status in order to observe any difference in the prognosis, gene expression and methylation levels in the two subgroups. In comparison with MSS group, MSI group had a significantly reduced expression of TNF in the PBMC samples. Although the MSI tumours are known to be immunogenic, such a finding has not been reported in the past and could represent a specific form of immune response in CRC. However, DNA methylation and survival did not correlate with MSI status in our study.

A high density of tumour-infiltrating FOXP3 + Tregs has been demonstrated to be associated with a poor outcome in a wide variety of malignancies including CRC (Curiel *et al*, 2004; Sato *et al*, 2005; Hiraoka *et al*, 2006; Kobayashi *et al*, 2007). Other studies have shown that it may be associated with favourable prognosis (Salama *et al*, 2009). The T-regulatory cells that are characterised by the expression of *FOXP3* have been shown to be capable of inhibiting tumour-associated antigen-specific immune responses in patients with CRC (Clarke *et al*, 2006). A significantly increased expression of FOXP3 by CRC cells has been demonstrated and was also found to be associated with poor prognosis (Kim *et al*, 2013). We have demonstrated that the *FOXP3* mRNA expression is enhanced in the tumour tissue. The expression was significantly higher in large-sized tumours (classified based on median tumour size). Hence, the expression of *FOXP3* can be considered to be associated with tumour growth and hence tumour progression. FOXP3 expression in tumour cells was found to be a strong predictor of metastatic disease and lymph node positivity in a study of human breast cancer patients (Merlo *et al*, 2009). *FOXP3* expression was also found to correlate with stage and lymph node metastasis in oesophageal squamous cancer (Xue *et al*, 2010). However, a differential expression of *FOXP3* in tumour tissue with respect to tumour size has not been demonstrated in the past. In our study, we have also clearly demonstrated that higher *FOXP3* expression in the PBMC was associated with significantly better OS and RFS. The significance of its association with better OS was also confirmed on multivariate Cox regression analysis. This finding could add to the prognostic significance of *FOXP3* expression in CRC.

Although MSPCR is a rapid and reliable measure of the methylation status in CpG island, the limitation is that it is not quantitative. Having identified a strong association of DNA methylation in the expression of *TNF* and *FOXP3* this could be taken forward by performing quantitative analysis with bisulfite pyrosequencing and demethylation experiments. Increased expression of *FOXP3* noted with respect to increasing size of the tumours will need to be confirmed with immunohistochemical studies. Considering the prognostic importance of *FOXP3* expression identified in our study further exploration of the canonical pathways of FOXP3 transcription in CRC will be necessary in the future.

CONCLUSION

Although it is already known that suppression of CMI is a feature in almost all cancers, our study has demonstrated specific alterations in the expression and methylation of CMI-associated immune genes that may offer novel immunomodulatory approaches in the management of CRC. We have demonstrated a possible role for cytokine genes *TNF* and *IFNG* in the CRC progression, with specific patterns of suppression noted in stage 4 and recurrent CRC. *FOXP3* appears to have a specific role in the progression of the primary tumour due to its association noted with tumour size. We have clearly demonstrated survival benefit in patients with higher PBMC *FOXP3* expression. In addition, we have shown that DNA methylation could play an important role in the regulation of *TNF* and *FOXP3*. Our novel findings will need further confirmation with the help of quantitative and pathway analyses.

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CONFLICT OF INTEREST

The authors declare no conflict of interest.

ETHICAL APPROVAL

South West London Research Ethics Committee (REC) 3, Charing Cross Hospital, London W6 8RF. REC Reference: 09/H0803/84. All persons gave their informed consent prior to their inclusion in the study.

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